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A Novel Galacto-Glycerolipid from *Oxalis corniculata* Kills *Entamoeba histolytica* and *Giardia lamblia*▽†

Dipak Manna,[1] Pradeep K. Dutta,[2] Basudeb Achari,[2] and Anuradha Lohia[1]*

Department of Biochemistry, Bose Institute,[1] and Division of Medicinal Chemistry, Indian Institute of Chemical Biology,[2] Kolkata, India

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***Oxalis corniculata* is a naturally occurring weed that has been used in traditional medicine for the cure of dysentery and diarrhea in India. One of the common causes of dysentery is due to infection by the protist pathogen *Entamoeba histolytica*. Bioactivity profiling of extracts from *O. corniculata* identified several compounds that showed antiamoebic activity in axenic cultures of *E. histolytica*. These were characterized by nuclear magnetic resonance, infrared, and mass spectrometry as (i) Oc-1, a mixture of saturated fatty acids C_{24} to C_{28}; (ii) Oc-2, a mixture of long-chain alcohols C_{18} to C_{28}; and (iii) Oc-3, a single compound that was a galacto-glycerolipid (GGL). Of the different compounds that were obtained, the strongest antiamoebic activity was found in GGL. The addition of GGL to *E. histolytica* xenic cultures containing other microbial flora from the large intestine did not affect its antiamoebic activity. Amoebicidal concentrations of GGL had no effect on intestinal microbial flora or on the mammalian cell line HEK-293. GGL was also found to be equally effective in killing another protist pathogen, *Giardia lamblia*, that causes diarrhea in humans. The importance of this study is based on the identification of novel natural products and the possibility of developing these compounds as active agents to treat at least two pathogenic parasitic intestinal infections endemic to tropical regions.**

Infection with *Entamoeba histolytica* is one of the most common types of intestinal infection in tropical areas of the developing world. Epidemiological data on the prevalence of this infection indicate that about 500 million people worldwide are infected, 90% of which are asymptomatic carriers, whereas the remaining 10% show serious intestinal and extraintestinal diseases such as colitis, dysentery, and amoebic liver abscess. Currently, metronidazole is the drug of choice for the treatment of amoebiasis and other gastrointestinal pathogens such as *Giardia* and *Trichomonas* spp. and *Helicobacter pylori*. Resistance to metronidazole is acquired easily by many intestinal bacteria and protists such as *Giardia intestinalis* and *Trichomonas vaginalis* (2) and is therefore a matter of serious concern. In addition, metronidazole is mutagenic in bacteria and carcinogenic in experimental mammalian models at high doses over long periods (13, 14). Prolonged use of metronidazole leads to unpleasant side effects, such as headache, vertigo, nausea, and metallic taste and sometimes to toxicity in the central nervous system and pancreatitis (12). Therefore, there is an urgent need to develop alternative drugs that do not have these undesirable side effects.

Several natural products with antiamoebic properties have been identified. These include emetine (9), tubulosine, cryptopleurine, berberine (19), and allicine (diallylsulfinate) (4, 5). However, either inefficacy in curing amoebiasis or the toxicity of these compounds has precluded their use as an alternative to metronidazole. The weed *Oxalis corniculata* is used in traditional medicine in India to cure dysentery, diarrhea, and skin diseases (11). In an effort to characterize active compounds from this plant, we used bioactivity profiling of its extracts and discovered a novel galacto-glycerolipid (GGL), in addition to two other compounds. We describe here the antiamoebic and antigiardial effect of the novel GGL and other compounds purified from *O. corniculata*.

MATERIALS AND METHODS

Plant material. Whole plants of *O. corniculata* were collected from the Botanical Garden, Botanical Survey of India (BSI), Kolkata, India, and authenticated at the BSI laboratories. A specimen voucher and herbarium of the plant material was deposited at the BSI.

Extraction and isolation of pure compounds. Activity-guided purification was performed for the isolation of active compounds from *O. corniculata* against *E. histolytica* as shown in a flow chart (Fig. 1A). Air-dried ground whole plants (2 kg) were extracted at room temperature with methanol, methanol-water (1: 1), and water consecutively using 6 liters of solvent each time. All of the extractions were repeated thrice. The extracts were concentrated under reduced pressure by using a rotary evaporator and lyophilized to test their activity.

The active methanol extract (200 g) was suspended in water (1 liter) and then partitioned with ethyl acetate (2 liters, three times) and *n*-butanol (2 liters, three times) successively. Each solvent fraction was combined and concentrated under reduced pressure by using a rotary evaporator and finally lyophilized. The ethyl acetate fraction (60 g) was found to be active and subsequently fractionated on silica gel columns (60-120 mesh) using a gradient of ethyl acetate and petroleum ether. Approximately 80 fractions (100 ml each) were collected. Fractions with similar thin-layer chromatography (TLC) profiles were pooled to obtain 15 fractions. Each of these 15 fractions was tested for antiamoebic activity *in vitro*. Among these, two fractions (fraction A [4 g] eluted with petroleum ether-ethyl acetate [8:2], and fraction B [2.5 g] eluted with petroleum ether-ethyl acetate [2:8]) showed growth inhibition of amoeba and were further purified over silica gel columns (100-200 mesh). Fraction A yielded two types of partially active compounds: (i) Oc-1, a mixture of long-chain saturated fatty acids C_{24} to C_{28} (70 mg), and (ii) Oc-2, a mixture of long-chain primary alcohols C_{18} to C_{28} (110 mg). Fraction B, upon further purification by flash column chromatography (230-400 mesh) and preparative TLC (Silica gel 60 F254; Merck) using a solvent system of petroleum ether-ethyl acetate (2:8 [vol/vol]), yielded a pure oily compound Oc-3 (50 mg).

Structure elucidation of active compounds Oc-1, Oc-2, and Oc-3. The identities and structures of Oc-1, Oc-2, and Oc-3 were determined by different methods as described below.

* Corresponding author. Mailing address: Department of Biochemistry, Bose Institute, Kolkata 700054, India. Phone: 91 33 25693224. Fax: 91 33 23553886. E-mail: amoeba@boseinst.ernet.in.

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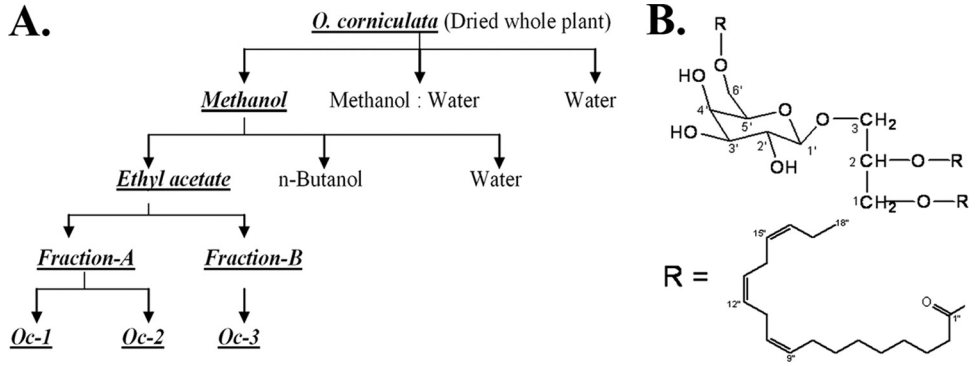

FIG. 1. Isolation of antiamoebic compounds from *O. corniculata*. (A) A flow chart shows the activity-guided purification of compounds from *O. corniculata.* The active extracts and/or compounds are in boldface and underlined. (B) Structure of GGL isolated from *O. corniculata*. Detailed ^{1}H and ^{13}C NMR spectral analysis showed that Oc-3 was a GGL, where a glycerol unit was linked to two fatty acyl chains (R, 18:3ω3) and a β-galactoside unit. A third 18:3ω3 fatty acyl chain (R) was linked to the C-6-OH of galactose.

(i) IR. Infrared spectrometry (IR) of Oc-1 and Oc-2 was carried out using KBr pellets and IR of Oc-3 was performed as neat on an FT/IR410 Jasco spectrophotometer.

(ii) NMR spectrometry. ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectrometry of Oc-1, Oc-2, and Oc-3 were recorded on a Bruker 300 or 500 MHz spectrophotometer using tetramethylsilane as an internal standard.

(iii) Mass spectrometry. Electrospray ionization mass spectra were recorded in a Waters Q-TOF Micro mass spectrometer.

(iv) GC-MS. Gas chromatography-mass spectrometry (GC-MS) and electrospray ionization-mass spectrometry (EIMS) were carried out on Shimadzu GC-MS-QP5050A and on JEOL JMS 600 instruments, respectively. Oc-1 and Oc-2 obtained from silica gel column chromatography were converted to methyl esters and acetate, respectively, and analyzed by GC-MS (7). Briefly, Oc-1 (20 mg) was dissolved in dry methanol (5 ml), a 10% solution of acetyl chloride in methanol (5 ml) was added, and the mixture was kept at room temperature overnight and then partitioned between water and ether. The ether layer was washed first with 10% $NaHCO_3$ solution (50 ml) and then with water, dried under nitrogen, and dissolved in chloroform for GC-MS. Oc-2 (60 mg) was dissolved in dry pyridine (2 ml), 0.5 ml of dry acetic anhydride was added, and the mixture was kept at room temperature overnight. A few drops of methanol were added to destroy free acetic anhydride, and the solvents were removed under vacuum with the addition of toluene. The acetylated alcohols were purified by column chromatography and identified by GC-MS.

Cell culture and maintenance. Trophozoites of *E. histolytica* strain HM1:IMSS were grown under axenic conditions in TYI-S-33 medium (8) at 37°C. *Giardia lamblia* Portland I trophozoites were cultured axenically at 37°C in filter-sterilized TYI-S-33 medium (10). Both amoeba and giardia trophozoites were routinely subcultured after 72 h. Trophozoites in the log phase of growth (48 h) were used in all experiments. Human embryonic kidney cells (HEK-293) were cultured at 37°C in an atmosphere of 5% CO_2 in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 100 U of penicillin/ml, and 100 μg of streptomycin/ml.

Rexenization of axenically cultivated *E. histolytica*. An axenic culture of *E. histolytica* was acclimatized initially in TYI-S-33–Robinson medium (1:1 [vol/vol]) (16) for 2 weeks, with substantial subculture after 72 h. Next, the *E. histolytica* cells were subcultured in complete Robinson medium supplemented with 10% adult bovine serum and rice starch. Large intestinal microbial flora from fresh fecal isolates (gut flora) were inoculated separately in Robinson medium, followed by incubation at 37°C. *E. histolytica* cells cultured in Robinson medium were chilled and harvested at 275 × *g* for 3 min. Then, 4×10^5 *E. histolytica* cells were added with gut flora in fresh Robinson medium, followed by incubation at 37°C. The cells were subcultured thrice a week, and trophozoites in the log phase of growth (48 h) were used for all experiments.

Bioactivity profiling of *O. corniculata* compounds. The inhibitory effect of the compounds was determined by a modification of the procedure of Neal (15) against *E. histolytica* and *G. lamblia* in axenically cultured TYI-S-33 medium. Briefly, *E. histolytica* and *G. lamblia* cells (4×10^4 cells/ml) were added to 10 ml of growth medium (with 10 or 1% serum, as specified in the text) in screw-cap culture tubes (Nunc, catalog no. 156758), followed by incubation at 37°C. Stock solutions of the compounds were prepared in 100% dimethyl sulfoxide (DMSO) and diluted in TYI medium. The maximum concentration of DMSO in each tube was <0.5%. Each test included metronidazole as standard drug and untreated controls (with or without 0.5% DMSO). Morphological changes were monitored under a light microscope, the trophozoites were counted in a hemocytometer, and the viability was determined by using a trypan blue exclusion assay (17).

Real-time microscopy. *E. histolytica* HM1:IMSS trophozoites (4×10^4 cells/ml) were plated on 35-mm glass culture dishes filled with 1% serum-supplemented growth medium. The culture dish was kept inside an incubator (Tempcontrol 37-2 Digital; Zeiss, Germany) at 37°C and under a 5% CO_2 flow system (PeCon GmbH, Erbach, Germany), which was fitted to an Axiovert 200M fluorescence microscope (Zeiss). Then, 2 μM FM4-64 (Molecular Probes) was added in culture. The cells were treated with DMSO or GGL and visualized immediately under a microscope by using a 40× oil immersion objective lens with an excitation at 510 nm. Time-lapse images were captured at 1-s intervals for the indicated times and then analyzed and further processed by using Axiovision v4.6 software (Zeiss).

Cell death assay. To determine the mode of cell death caused by GGL, *E. histolytica* cells were treated with different concentrations of GGL in 1% serum-supplemented medium for 1 h. Solvent-treated control and GGL-treated axenically grown amoeba cells—with or without the addition of bovine serum albumin (BSA) and *E. coli* at different concentrations (10^6 to 10^8)—and axenically grown (with gut flora) amoeba cells were collected after incubation and washed twice with 1× phosphate-buffered saline (PBS; pH 7.4). Cells were suspended in 100 μl of annexin V binding buffer and labeled with annexin V (Molecular Probes) and propidium iodide (PI) at 0.2 mg/ml. Gut flora and *E. coli* were treated similarly. The cells were analyzed by flow cytometry (FACSCalibur; Becton Dickinson). Forward scatter and side scatter (FSC/SSC) settings for amoeba cells were different from those used for bacterial cells.

Cytotoxicity assay. HEK-293 cells were treated with Oc-1, Oc-2, and Oc-3 to test the toxicity of these compounds for mammalian cells. The cell viability was assessed by using an MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] assay. HEK-293 cells were cultured in DMEM in 96-well microtiter plates and incubated at 37°C in an atmosphere of 5% CO_2. In each well 10^4 cells were added. Oc-1, Oc-2, and Oc-3 were added at different concentrations (25, 50, and 100 μg/ml), followed by incubation for 24 h in complete medium at 37°C. After incubation, the culture medium was decanted and once washed with 1× PBS. Then, 10 μl of MTT solution (Roche) was added in 100 μl of PBS in each well, and the plates were incubated for an additional 4 h. After that, 100 μl of solubilization buffer was added to each well to solubilize the formazan, and the plates were incubated at 37°C overnight. The absorbance was then measured at 595 nm. The results were expressed as a percentage of the viable cells.

RESULTS

Activity-guided purification and structural characterization of Oc-1, Oc-2, and Oc-3. Activity-guided purification of *O. corniculata* organic extract by silica gel chromatography yielded two enriched fractions, Oc-1 and Oc-2, and one pure compound, Oc-3, as described here and as shown in the flow chart (Fig. 1A). Elucidation of the structures and identities of

TABLE 1. Fatty acids present in Oc-1 and long-chain primary alcohols present in Oc-2 and their relative amounts[a]

Fatty acid or alcohol	Relative amt (%)
Fatty acids	
Lignoceric acid ($C_{24:0}$)	58
Hexacosanoic acid ($C_{26:0}$)	26
Octacosanoic acid ($C_{28:0}$)	16
Alcohols	
1-Octadecanol ($C_{18}H_{38}O$)	2
1-Docosanol ($C_{22}H_{46}O$)	3
1-Pentacosanol ($C_{25}H_{52}O$)	3
1-Heptacosanol ($C_{27}H_{56}O$)	3
1-Octacosanol ($C_{28}H_{58}O$)	89

[a] The fatty acid mixture obtained from Oc-1 by silica gel column chromatography was converted to its methyl ester and analyzed by GC. The alcohol mixture obtained from Oc-2 by silica gel column chromatography was converted to acetate and analyzed by GC.

TABLE 2. IC_{50}s of different compounds isolated from *O. corniculata* against *E. histolytica* and *G. lamblia*

Compound	Mean IC_{50} (μg/ml) ± SD[a]	
	E. histolytica	*G. lamblia*
Oc-1	24 ± 0.2	184 ± 0.3
Oc-2	35 ± 0.03	206 ± 0.4
Oc-3 (GGL)	15 ± 0.2	3.7 ± 0.6
Metronidazole	1 ± 0.1	2 ± 0.2

[a] IC_{50} values were determined after the addition of different concentrations of the compounds to cells in 10% serum-containing growth medium for 24 h each. Experiments were performed in triplicates ($n = 3$; $P \leq 0.05$).

the compounds in Oc-1, Oc-2, and Oc-3 were carried out by spectral analyses as described below.

Oc-1. The IR spectrum showed a broad carboxyl absorption at 1,712 cm^{-1}, along with O-H stretching bands at 3,500 to 2,500 cm^{-1} overlapping the C-H stretching bands. This indicated that the compound might contain carboxyl group(s). The ^1H NMR spectrum showed a triplet at δ 0.88 (J = 6.4 Hz) for the terminal methyl, a broad singlet integrating for approximately 40 protons at δ 1.25 for methylene groups, a multiplet at δ 1.63 for the methylene β to the carboxyl, and a triplet at δ 2.35 (J = 7.5 Hz) for the α-methylene. There was no signal in the δ 5 region, eliminating the presence of unsaturation. This suggested that the compound might be a saturated fatty acid or a mixture. The electrospray ionization mass spectrum also showed that this is mainly a mixture of saturated fatty acids (C_{24} to C_{28}). GC-MS analysis of its methyl ester confirmed that it is a mixture of three long-chain saturated fatty acids, viz. lignoceric acid, hexacosanoic acid, and octacosanoic acid, with relative abundances as shown in Table 1.

Oc-2. The spectrometric data of Oc-2 indicated that this fraction was a mixture of long-chain saturated alcohols. The IR spectrum showed a broad -OH stretching band at 3,348 cm^{-1}, which indicated that the compound might contain hydroxyl group(s). The ^1H NMR spectrum showed a triplet at δ 0.88 (J = 6.4 Hz) for the terminal methyl group, a broad singlet at δ 1.25 integrating for approximately 38 protons for methylene groups, and a triplet at δ 3.64 (J = 6.6 Hz) for a $-CH_2-OH$ group. The ^1H NMR spectrum of the acetylated Oc-2 showed peaks at δ 0.88 (t, J = 6.5 Hz, $-CH_3$), 1.25 [s, $-(CH_2)_n-$], 2.04 (s, $-OCOCH_3$), and 4.05 (t, J = 6.6 Hz, $-CH_2-CH_2-OCOCH_3$). These data confirmed that the compound Oc-2 contains long-chain primary alcohols. GC-MS analysis showed that Oc-2 contained 1-octadecanol ($C_{18}H_{38}O$), 1-docosanol ($C_{22}H_{46}O$), 1-pentacosanol ($C_{25}H_{52}O$), 1-heptacosanol ($C_{27}H_{56}O$), and 1-octacosanol ($C_{28}H_{58}O$) in different amounts (Table 1). The predominant alcohol (89%) is 1-octacosanol ($C_{28}H_{58}O$).

Oc-3. The IR spectrum showed a broad absorption centered at 3,437 cm^{-1} attributed to hydroxyl group(s). A sharp peak at 3,010 cm^{-1} indicated the presence of unsaturation. The strong absorption at 1,739 cm^{-1} pointed to the existence of ester linkage. The IR spectrum suggested that the compound may be an ester of an unsaturated fatty acid. The ^1H NMR spectrum of Oc-3 showed a sharp triplet at δ 0.98 (J = 7.8 Hz) commensurate with the methyl signal of ω:3 fatty acid derivatives. It also showed signals at δ 2.08 (*m*, $-CH_2-CH=CH-$), 2.34 (m, $-CH_2-COO-$), 2.81 (m, $-CH=CH-CH_2-CH=CH-$), and 5.36 ($-CH=CH-$), with approximate integration ratio of 9:6:9:11, supporting the presence of a Δ^3 acid derivative as the major component. Two other isolated spin systems identified the presence of a glyceryl and a hexosyl unit. A 1H doublet at δ 4.22 (J = 7 Hz) could be ascribed to the anomeric proton of the hexose unit (β). It also showed signals at δ 4.2 to 4.3 corresponding to 6′-H_2, the distinctly downfield shift pointing to the presence of an ester linkage at this position. The structural conclusions were corroborated by spectral analysis of the Oc-3 triacetate. The ^1H NMR spectrum of Oc-3 triacetate showed signals for three acetyl methyl groups, and the overlapping 2′ and 3′ proton signals of the original sample were fully resolved.

Detailed NMR assignments were derived from two-dimensional NMR spectrometric analysis (^1H-^1H COSY, NOESY, ^1H-^{13}C HMQC, and ^1H-^{13}C HMBC) of Oc-3 and its acetate, leading to the final structure (see Table S1 in the supplemental material). The spectroscopic data suggest that Oc-3 was a single compound, where a glycerol unit was linked to two fatty acyl chains (18:3ω3) and a β-galactoside unit. A third 18: 3ω3 fatty acyl chain was linked to the C-6-OH of galactose (Fig. 1B). This compound was named galacto-glycerolipid (GGL). The spectral data closely resembled those reported for similar galactosyl glycerolipids (1, 3).

***In vitro* antiamoebic and antigiardial activity of different compounds isolated from *O. corniculata*.** In order to compare the activity of Oc-1, Oc-2, and Oc-3, we added each of these in different concentrations to *E. histolytica* growing in axenic cultures and inhibition of cell viability was monitored by trypan blue assay. The three components—Oc-1, Oc-2, and Oc-3—caused decreases in *E. histolytica* viability in a concentration-dependent manner, with 50% inhibitory concentrations (IC_{50}s) of 24 ± 0.2, 35 ± 0.03, and 15 ± 0.2 μg/ml, respectively, after 24 h of treatment (Table 2). These compounds also demonstrated different efficacies in killing amoeba cells over time. The Oc-1 and Oc-2 fractions initially showed significant cell killing 24 h after addition to axenic cultures (33 to 38% cells were viable), but no further killing was observed after 24 h, and the cell viability increased to 48 to 57% after 72 h (Fig. 2A). On the other hand, Oc-3/GGL-treated cells showed complete loss of viability within 48 h accompanied with aggregation and lysis. The standard antiamoebic drug metronidazole showed the expected loss of viability in 48 h. Bright-field microscopy of Oc-

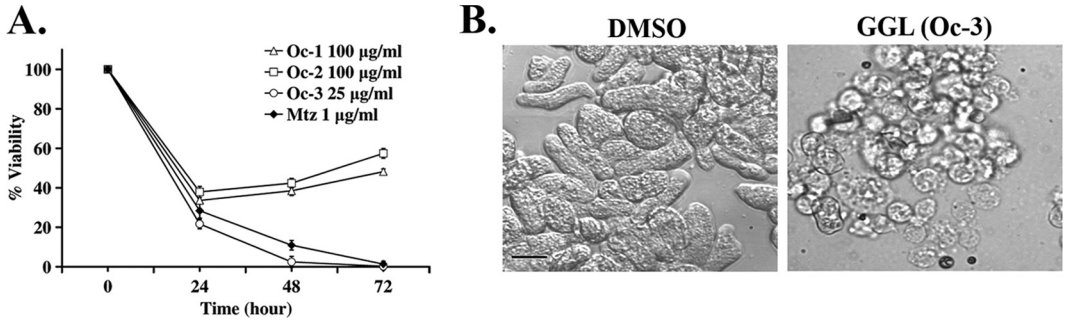

FIG. 2. Amoebicidal activity of different compounds isolated from *O. corniculata* against *E. histolytica*. (A) Axenic cultures of *E. histolytica* were treated with purified Oc-1 (△), Oc-2 (□), and Oc-3/GGL (○) of *O. corniculata* and metronidazole (Mtz [◆]) as a standard for 0, 24, 48, and 72 h. At different time periods, the numbers of cells were counted, and the viability was determined by using a trypan blue exclusion assay. The results from three experiments are shown as the percent viability (± the standard deviation) with respect to the solvent control. (B) The aggregation and lysis of *E. histolytica* cells caused by incubation with GGL (Oc-3) was monitored under a microscope. *E. histolytica* cells were treated with 25 μg of GGL (Oc-3)/ml and 0.5% DMSO as a solvent control. After 24 h, the cells were observed under microscope, and photographs were obtained by using a 20× objective lens. Bar, 20 μm.

3/GGL-treated *E. histolytica* confirmed the detachment and lysis of cells (Fig. 2B).

Since *O. corniculata* was traditionally used to treat diarrhea and dysentery, we tested the ability of *Oxalis* compounds to kill *G. lamblia*, another intestinal protozoan pathogen that causes diarrhea. The GGL (Oc-3) showed strong antigiardial activity, with an IC_{50} of 3.7 ± 0.6 μg/ml (Table 2), whereas the mixture of saturated fatty acids (Oc-1) and long-chain alcohols (Oc-2) showed mild antigiardial activity with very high IC_{50}s of 184 ± 0.3 and 206 ± 0.4, respectively, after 24 h of treatment (Table 2). A total of 87% of Oc-3-treated *Giardia* cells were dead after 24 h, and the rest were dead within 72 h after the addition of GGL. In contrast, the Oc-1 and Oc-2 fractions at 100 μg/ml showed only 10 to 20% killing by 24 h after addition of GGL, with no increase in cell killing up to 72 h (Fig. 3A). Killing of *G. lamblia* by metronidazole (1 μg/ml) was comparable to that caused by GGL. Bright-field microscopy of Oc-3/GGL-treated *G. lamblia* confirmed the detachment and lysis of cells (Fig. 3B). Compared to Oc-1 and Oc-2, GGL was the strongest antiamoebic and antigiardial compound isolated from *O. corniculata.* In subsequent experiments, we focused on identifying parameters that enhanced the activity of GGL.

***E. histolytica* and *G. lamblia* cells were more susceptible to GGL when cultured in medium at lower serum concentrations.** Bovine serum contains several lipid-binding proteins and factors, including large amounts of BSA. BSA is known to bind free fatty acids (18) and therefore could reduce the availability of GGL to amoeba and giardia trophozoites in culture. In order to test this hypothesis, the serum concentration of the growth medium was reduced to 1% compared to 10% in regular growth medium. It was observed that neither amoeba nor giardia viability was affected by up to 12 to 16 h of reduced serum in the growth medium (data not shown). GGL was added at different concentrations to *E. histolytica* or *G. lamblia* cells in TYI medium supplemented with 1 or 10% serum, followed by incubation at 37°C. The cells were harvested after 8 h, and viability was determined by using the trypan blue exclusion assay. Our results show that GGL (Fig. 4A) killed both amoeba and giardia cells in 1% serum much faster than cells in 10% serum-containing medium. In contrast, the mixture of long-chain fatty acids (Oc-1) and long-chain alcohols (Oc-2) did not show enhancement in their activity at low serum concentrations (data not shown).

Both Oc-1 and Oc-2 at 100 μg/ml showed mild antiamoe-

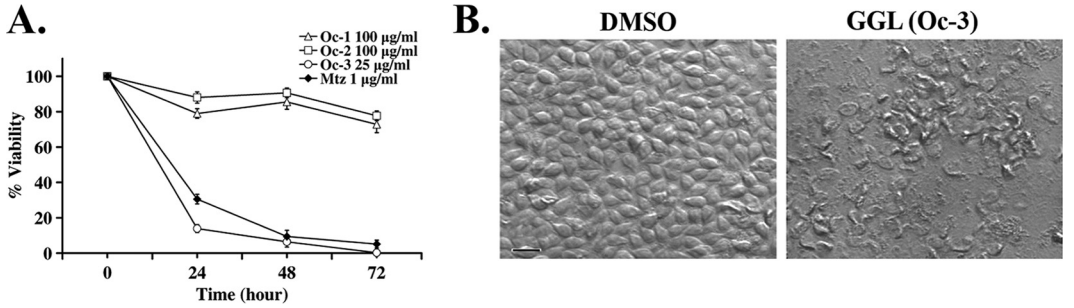

FIG. 3. Antigiardial activity of different compounds isolated from *O. corniculata* against *G. lamblia*. (A) Axenic cultures of *G. lamblia* were treated with purified Oc-1 (△), Oc-2 (□), and Oc-3/GGL (○) of *O. corniculata* and metronidazole (Mtz [◆]) as a standard for 0, 24, 48, and 72 h. At different time periods, the numbers of cells were counted, and the viability was determined by using a trypan blue exclusion assay. The results from three experiments are shown as the percent viability (± the standard deviation) with respect to the solvent control. (B) The aggregation and lysis of *G. lamblia* cells caused by incubation with GGL (Oc-3) was monitored under a microscope. *G. lamblia* cells were treated with 25 μg of GGL (Oc-3)/ml and 0.5% DMSO as a solvent control. After 24 h, the cells were observed under a microscope, and photographs were obtained by using a 20× objective lens. Bar, 20 μm.

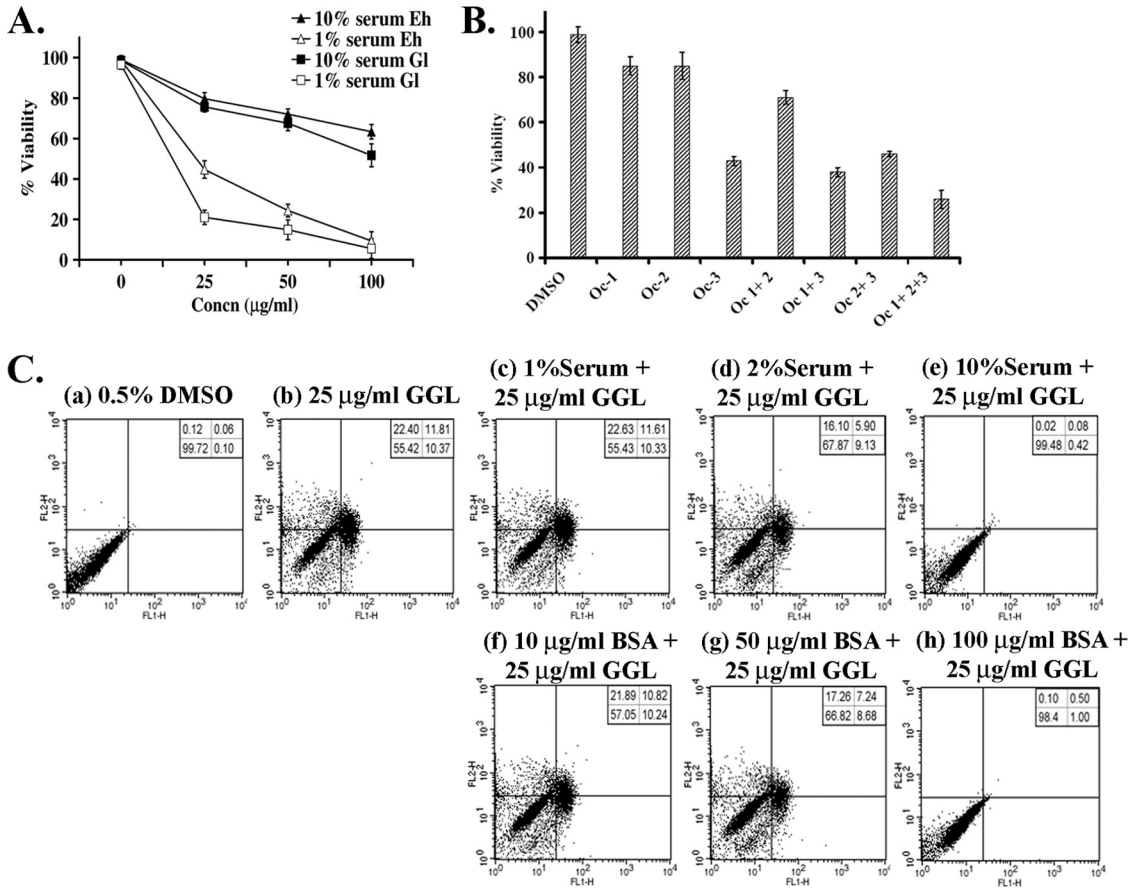

FIG. 4. *E. histolytica* and *G. lamblia* cells were more susceptible to GGL when cultured in medium with a low serum concentration. (A) *E. histolytica* (triangles) and *G. lamblia* (squares) cells were incubated in 1% serum- or 10% serum-containing medium and treated with different concentrations of GGL for 8 h. Solid symbols indicate medium containing 10% serum, and open symbols indicate medium containing 1% serum. After 8 h, the numbers of cells were counted, and the viability was determined by using a trypan blue exclusion assay. The results from three experiments are shown as the percent viability (± the standard deviation) with respect to the solvent control. (B) *E. histolytica* cells were cultured in TYI medium supplemented with 1% serum. Cells were treated with Oc-1, Oc-2, and Oc-3 individually or in combination with one another. Oc-1 and Oc-2 were used at 100 μg/ml, and Oc-3 was used at 25 μg/ml. DMSO (0.5%) was used as a solvent control, and the viability was determined by using a trypan blue exclusion assay after 8 h. The results from two experiments were shown as the percent viability (± the standard deviation). (C) *E. histolytica* cells were incubated in TYI medium and treated for 1 h with 0.5% DMSO (a), 25 μg of GGL/ml (b), 25 μg of GGL/ml and 1% serum (c), 25 μg of GGL/ml and 2% serum (d), 25 μg of GGL/ml and 10% serum (e), 25 μg of GGL/ml and 10 μg of BSA/ml (f), 25 μg of GGL/ml and 50 μg of BSA/ml (g), or 25 μg of GGL/ml and 100 μg of BSA/ml (h). The cells were stained with annexin V and PI to detect the percentage of apoptotic and/or necrotic cells by flow cytometry. Annexin V and PI were labeled as FL1-H and FL2-H, respectively. Apoptotic cells were visualized in the upper and lower right-hand quadrants of the dot plot. The percentage of cells in each quadrant is represented in the inset box in the dot plot.

bic activity (10 to 20% cell killing in 8 h) when added individually to amoeba trophozoites. The two fractions together did not show a significant increase in activity. On the other hand, 25 μg of GGL/ml killed ca. 60% of the cells within 8 h. The addition of 100 μg of Oc-1/ml with 25 μg of GGL/ml enhanced the antiamoebic effect of GGL marginally, whereas the addition of Oc-2 did not show any significant increase. However, the addition of both Oc-1 and Oc-2 together with GGL enhanced the antiamoebic effect of Oc-3 so that cell viability was reduced from 40% (GGL alone) to 25% (Fig. 4B). It is likely that these fractions together act synergistically to kill amoeba. The addition of Oc-1 and Oc-2 to Oc-3/GGL did not show any increase in giardicidal activity compared to Oc-3/GGL alone (data not shown). Thus, the addition of Oc-1 and Oc-2 with GGL yielded increased killing of amoebas but not giardias.

Within 1 h of addition of GGL, apoptotic cell death was seen in *E. histolytica* cells incubated in low serum (0 to 2%)-containing medium (Fig. 4C), while cells in 10% serum-containing medium were normal. Supplementation with BSA (100 μg/ml) in serum-free medium was sufficient to protect the cells from the killing by GGL (Fig. 4C). Lower concentrations of BSA (10 to 50 μg/ml) showed a dose-dependent protective effect (Fig. 4C). Our results suggest that the presence of high concentrations of BSA in 10% serum-containing medium possibly sequesters GGL and reduces the availability of the compound to amoeba or giardia trophozoites. The presence of BSA and other lipophilic compounds in serum may be the reason why

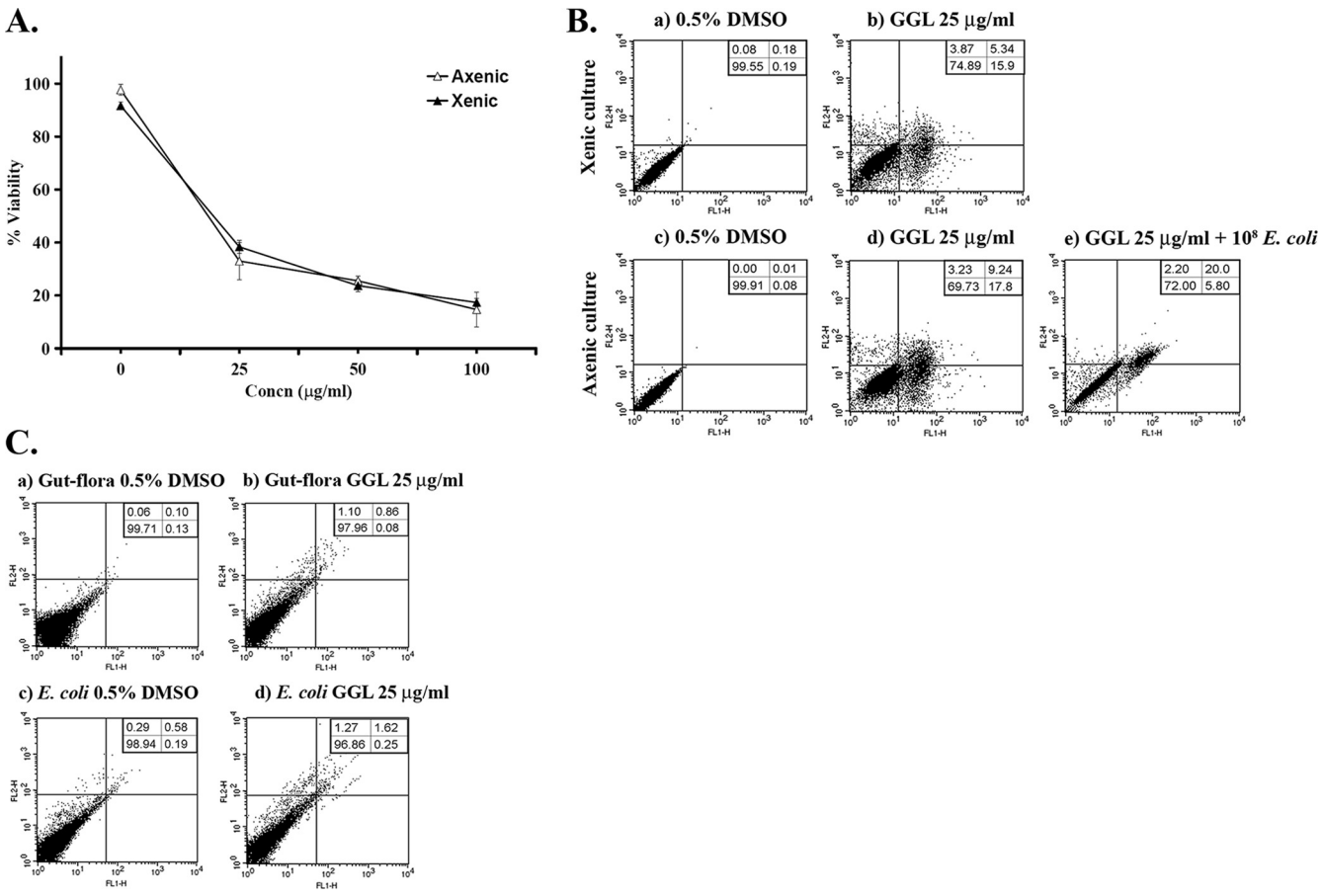

FIG. 5. Comparison of GGL activity against *E. histolytica* under xenic and axenic growth conditions. (A) GGL is active against *E. histolytica* in the presence of bacteria or intestinal microbial flora. Xenic and axenic cultures of *E. histolytica* were treated with GGL at different concentrations (0, 25, 50, and 100 μg/ml) for 24 h. After 24 h, the numbers of cells were counted, and the viability was determined by using a trypan blue exclusion assay. Filled symbols (▲) indicate xenic cultures, and empty symbols (△) indicate axenic cultures. The results from three experiments are shown as the percent viability (± the standard deviation) with respect to the solvent control. (B) GGL induces apoptotic cell death of *E. histolytica* in both xenic and axenic conditions. *E. histolytica* cells cultured in both xenic and axenic conditions were resuspended in Robinson and in TYIS-33 medium supplemented with 1% serum, respectively. Cells were treated with 0.5% DMSO (a and c) and 25 μg of GGL/ml (b and d) for 1 h. (e) Axenic culture of *E. histolytica* treated with 25 μg of GGL/ml in the presence of 10^8 *E. coli*. (*E. coli* cells were added at different concentrations ranging from 10^6 to 10^8 cells. However, no difference was seen for the different cell numbers, so only the results for *E. coli* 10^8 cells are shown.) Cells were labeled with annexin V and PI to detect the percentage of apoptotic or necrotic cells by flow cytometry. Apoptotic cells were visualized in the upper and lower right-hand quadrants of the dot plot. The percentage of cells in each quadrant is represented in the inset box in the dot plot. Annexin V and PI were labeled as FL1-H and FL2-H, respectively. (C) GGL does not induce apoptotic cell death in *E. coli* or gut flora. Gut flora and *E. coli* were treated for 1 h with 0.5% DMSO (a and c) and with 25 μg of GGL/ml (b and d), respectively. Cells were labeled with annexin V and PI to detect the percentage of apoptotic or necrotic cells by flow cytometry. Apoptotic cells are visualized in the upper and lower right-hand quadrants of the dot plot. The percentage of cells in each quadrant is represented in the inset box in the dot plot. Annexin V and PI were labeled as FL1-H and FL2-H, respectively. The FSC/SSC settings for bacteria and gut flora were similar to each other but different from those used for *E. histolytica.*

GGL takes longer to kill these protozoa in 10% serum-supplemented growth medium.

GGL is active against *E. histolytica* in the presence of bacteria or gut flora. For the therapeutic use of GGL, it must have activity in the presence of bacteria or gut flora, which are present with intestinal parasites in the human host. In order to test the efficacy of GGL in the presence of gut flora, we rexenized *E. histolytica* trophozoites with freshly isolated gut flora as described above. The activity of GGL in killing *E. histolytica* grown under axenic and xenic conditions was compared, and it was observed that GGL killed amoeba trophozoites with similar IC_{50}s in the two growth conditions (Fig. 5A). Thus, gut flora did not inhibit the activity of GGL on *E. histolytica.* In addition, GGL-induced apoptotic cell death was similar for *E. histolytica* grown in both xenic (Fig. 5Ba and b) and axenic (Fig. 5Bc and d) conditions. In addition to our experiments with gut flora, we also added *E. coli* cells at different concentrations to axenically growing *E. histolytica* and observed that these bacteria did not affect the antiamoebic activity of GGL (Fig. 5Be). Since *E. coli* and/or gut flora are smaller in size than amoeba cells, they were detected at different flow cytometric settings (Fig. 5C). We treated the gut flora (Fig. 5Ca and b) and *E. coli* (Fig. 5Cc and d) with GGL and observed that GGL did not induce apoptosislike death in *E. coli* or gut flora and that 96 to 98% of the cells were viable in each case (Fig. 5C). Thus, GGL does not affect bacteria and other gut microbes, and its amoe-

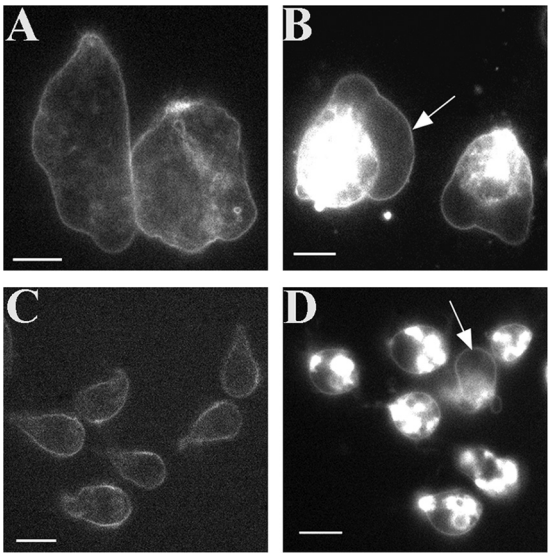

FIG. 6. GGL induces membrane blebbing and causes cell lysis. *E. histolytica* and *G. lamblia* cells were treated with 0.5% DMSO (A and C) or 25 μg of GGL/ml (B and D) in low-serum medium (1%) and stained with FM4-64 in culture. FM4-64 stained the cell membrane of intact *E. histolytica* and *G. lamblia* (A and C) treated with DMSO for 30 min. In contrast, 30 min after addition of GGL, membranous blebs are seen (B and D, arrows), and FM4-64 stains the intracellular membranous components and accumulates in both amoeba and giardia cells. Bar, 10 μm.

bicidal or giardicidal effect is not inhibited in the presence of bacteria or gut flora.

GGL induces membrane blebbing and causes cell lysis. Since GGL induces apoptotic cell death very soon after its addition, we investigated the real-time effect of adding GGL to *Entamoeba* and *Giardia* trophozoites using FM4-64 dye. The membrane-selective FM dyes belong to a class of amphiphilic styryl dyes developed by Bolte et al. (6). Due to its amphiphilic nature, FM4-64 is unable to cross the cell membrane and binds to the outer leaflet of the bilayer, where it fluoresces in the hydrophobic environment. Therefore, intracellular fluorescence can only be seen if the cell membrane is ruptured when the dye can enter the cell. Untreated amoeba and giardia cells showed FM4-64 fluorescence in the cell membrane alone (Fig. 6A and C). At 30 min after the addition of GGL, both amoeba and giardia cells released membranous blebs, followed by intracellular accumulation of the dye (Fig. 6B and D). Finally, both amoeba and giardia cells detached from the substratum, and gross lysis was observed. The addition of metronidazole to FM4-64-stained cells did not show similar membranous blebbing or cell lysis (data not shown). However, cells became rounded and detached from the substratum.

Antiprotozoal compounds from *Oxalis* do not show any cytotoxic effect on HEK-293 cells. GGL (Oc-3) inhibits the growth of *E. histolytica* and *G. lamblia*, with IC_{50}s of 15 ± 0.2 and 3.7 ± 0.6 μg/ml. At these inhibitory concentrations, GGL showed no significant toxic effect on HEK-293 cells (Fig. 7). At 8- to 10-fold higher concentrations (100 μg/ml), GGL showed ca. 15% growth inhibition of HEK-293 cells, whereas Oc-1 and Oc-2 showed no significant toxicity at 100 μg/ml.

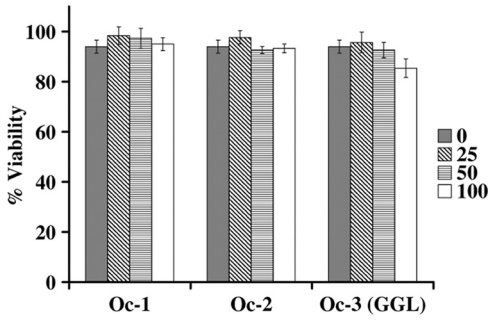

FIG. 7. Antiprotozoal compounds from *O. corniculata* do not show any cytotoxic effect on HEK-293 cells. HEK-293 cells were treated with the purified compounds Oc-1, Oc-2, and Oc-3 at 0 to 100 μg/ml for 24 h. The viability was determined by using an MTT assay. The results from three experiments are shown as the percent viability (± the standard deviation) with respect to the solvent control.

DISCUSSION

O. corniculata is known to cure dysentery, diarrhea, and skin diseases (9), but the active constituents were not identified earlier. The results of the present study suggest that purified compounds of *O. corniculata* were effective in killing *E. histolytica* and *G. lamblia* trophozoites. The active compounds we describe here are a group of long-chain saturated fatty acids (C_{24} to C_{28}), long-chain primary alcohols (C_{18} to C_{28}) and, most importantly, a novel galacto-glycerolipid (GGL). It is remarkable that biochemical fractionation of the organic extracts of *O. corniculata* led to identification of a single compound with strong activity against two intestinal protozoan parasites. The saturated fatty acids and long-chain alcohols showed mild antiamoebic and antigiardial activity. Although long-chain alcohols are known to have detergent activity that can disrupt cell membranes, these compounds were not strong antiamoebic or antigiardial agents. On the other hand, GGL was a potent compound that killed both amoebas and giardias at low concentrations. The data obtained from real-time microscopy showed that addition of GGL led to almost immediate lysis of both *Giardia* and *E. histolytica* cells. Although GGL is able to kill a significant number of *E. histolytica* and *G. lamblia* trophozoites in 10% serum-containing medium, its effect was substantially enhanced in reduced serum.

In the light of our results, we conclude that the novel GGL isolated from *O. corniculata* is a strong candidate for development as a therapeutic agent to eliminate two intestinal pathogens. First, although GGL shows strong activity against amoebas and giardias in the presence of 10% serum, GGL shows even higher activity in low serum concentrations, and the environment of the large intestine is known to have negligible serum. Second, GGL was able to kill amoebas with equal efficacy in the presence of bacteria or intestinal microbial flora and did not affect their viability. Finally, the mammalian cell line HEK-293 showed no appreciable loss of cell viability after treatment with GGL at amoebicidal and giardicidal concentrations. The development of such a natural product is especially important for areas in tropical countries where these diseases are endemic and where the weed is abundantly available, eaten as part of the diet, and would likely have fewer toxic effects than currently available drugs.

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A patent describing the identification of *O. corniculata* extracts that kill *E. histolytica* and *G. lamblia* has been filed in the Office of Controller General of Patents, Designs, and Trademarks, India (1686/KOL/2008). The plant *O. corniculata* was one of the six plants suggested by the late K. Nambiyar, Research Director of Arya Vaidyashala, Kotakkal, Kerala, India, for its use as an ayurvedic remedy for diarrhea and dysentery.

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